IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Martin E. Davis, *et al.*

Serial No. 09/702,068

Filed October 24, 2001

For: ENZYMATIC TREATMENT OF WHEY PROTEINS FOR THE PRODUCTION OF ANTIHYPERTENSIVE PEPTIDES AND THE RESULTING PRODUCTS

Group Art Unit 1651

Examiner
Michael Meller

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION OF ANAND RAO, Ph.D.

not signed - not comparing art used by examiner

I, ANAND RAO, am an inventor of the invention described in the above-identified U. S. Patent Application, and declare as follows:

not applied ref *

I have reviewed the methodology described in the Japanese patent (JP 04282400) and compared it to the subject matter taught and claimed in the present application. Based on this review and comparison, I draw several significant conclusions, which I detail below.

different ref. from applied ref.

(1) A Comparison of the Process Of JP 04282400 to That of the Invention Shows the Invention to be More Practical.

In Example 1 of JP 04282400, the inventors describe their method of production of a highly-purified ACE-inhibitory peptide. According to their description, they obtained 100 mg of the purified peptide as powder from 100 grams of cheese whey powder.

Cheese whey powder has approximately 12 grams of protein per 100 grams of powder. According to the disclosure, JP 04282400 reports a product recovery of less than 1% (0.83%) of

the available protein in the peptide form. Such extreme low yield of components in the form of functional food ingredient is not economical.

Assuming doses of 500 mg per kg body weight as set forth in JP 04282400, a 70 kg man would need to supplement his diet with about 35 grams of this product. Significantly, this would require the use of whey sufficient to supply over 4 kg of whey protein.

The process of the invention, on the other hand, will enable a recovery of a major amount of the available protein in the peptide form. As such, it is an ingredient produced in large volumes and has a proven marketability and commercial viability.

(2) Reported Results of Animal Investigations for the Products of JP 04282400 Show a Product of Far Less Activity Than Similar Tests Run Utilizing Products of the Present Invention.

DESCRIPTION OF THE METHOD:

The Japanese inventors, in their patent number JP 04282400, describe the efficacy of the ACE-inhibitory peptide in 12-week old male spontaneously hypertensive rats (SHR).

In one example (Test 2) the inventors evaluated the efficacy of various dosages (0, 125, 250, and 500 mg peptide per kg body weight). The systolic blood pressure (SBP) was measured by tail-cuff method after 6 hours of feeding and reported as mm Hg.

In another example (Test 3), SHR rats were force-fed 1000 mg of the peptide material per kg body weight. Physiological saline was used as a control. Blood pressure was measured during a 24-hour period by tail-cuff method and was reported as mm Hg.

We performed similar testing on the hydrolyzed whey protein isolate (BioZate 1[1]) using 23-week old male SHR. The dosages of BioZate 1 evaluated were 30, 75, and 150-mg powder

[1] Preparation of BioZate 1 is described in section 4, below.

per kg body weight. The mean arterial pressure (MAP) was measured after 6 hours and recorded automatically by intravascular catheter connected to a computer.

In our *in vivo* study using SHR, 150 mg of BioZate 1 per kg body weight was fed by gastric intubation. The mean arterial pressure was measured during a 7-hour period and recorded automatically by intravascular catheter connected to a computer.

RESULTS AND DESCRIPTION:

1. **Dosage:**

The following table compares the results reported in JP 04282400 and our results.

Data Reported in JP 04282400	
Peptide (mg/kg body weight)	Systolic Pressure (mm Hg)
0	222.3 ± 2.0
125	218.1 ± 7.0
250	216.5 ± 3.7
500	201.6 ± 3.5 *

* Significantly different from zero dose (P <0.01)

Data from Our Study	
BioZate 1 (Hydrolyzed Whey Protein Isolate) (mg/kg body weight)	Mean Arterial Pressure (mm Hg)
0	182.7 ± 21.2
30	144.6 ± 15.5
75	135.0 ± 9.7 *
150	130.3 ± 7.8 *

* Significantly different from zero dose (P < 0.01)

It is clear from the above data that even though the procedure of JP 04282400 had purified the specific ACE-inhibitory peptide, the biological efficacy of that process is much lower compared to that of BioZate 1. Their data indicate that a significant reduction in the systolic blood pressure (approximately 21mm reduction) was found only at a dose of 500 mg

peptide per kg body weight. This dose is approximately 6.7 times greater than the dose of BioZate 1 required to notice a reduction in the mean arterial pressure.

Monitoring of efficacy during several hours:

The following tables contrast the systolic blood pressure (SBP) as disclosed in JP 04282400 and the mean arterial pressure (MAP) as measured in our study. The mean arterial pressure is a mathematically derived value that takes in to account both the systolic and diastolic pressures, and is considered as a better representation of the blood pressure. JP 04282400 does not report any data on the diastolic pressures.

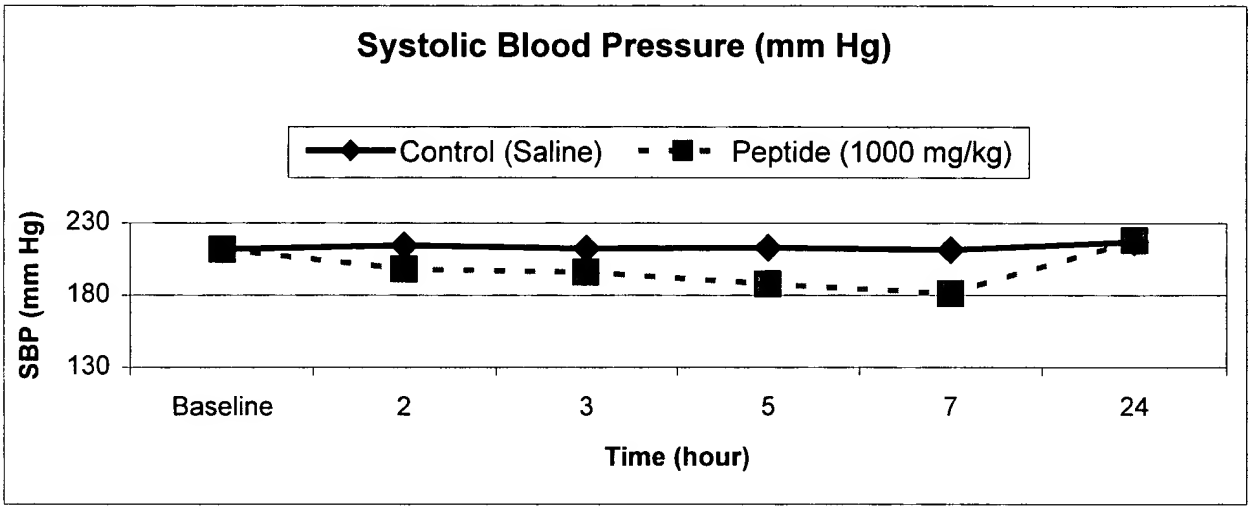
The dosage of the purified peptide used in the Japanese patent was 1000 mg/kg body weight. The dosages used in our study were 75 mg and 150 mg/kg body weight.

Data from the Japanese patent		
	Systolic Blood Pressure (mm Hg)	
	Control (Saline)	Peptide (1000 mg/kg)
Baseline	211.8 ± 3.5	211.8 ± 3.5
2 hours after feeding	214.4 ± 2.9	198.0 ± 7.2
4 hours after feeding	212.1 ± 2.3	195.8 ± 8.0
5 hours after feeding	212.9 ± 3.6	187.8 ± 6.8
7 hours after feeding	211.2 ± 3.6	181.1 ± 2.7
After 24 hours	217.0 ± 3.7	218.0 ± 3.5

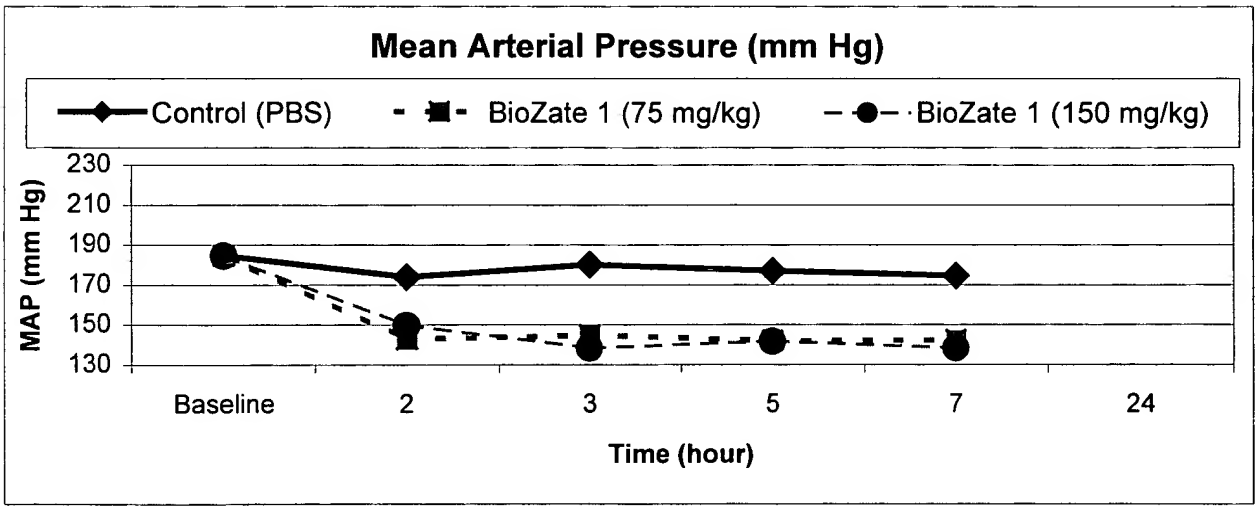
Data from Our study			
	Mean Arterial Pressure (mm Hg)		
	Control (PBS)	BioZate 1 (75 mg/kg)	BioZate 1 (150 mg/kg)
Baseline	184.6 ± 10.9	180.5 ± 6.1	167.0 ± 15.1
2 hours after feeding	173.9 ± 9.6	142.7 ± 14.7	149.8 ± 9.7
4 hours after feeding	180.1 ± 21.1	144.8 ± 11.7	138.3 ± 10.2
5 hours after feeding	176.9 ± 21.9	142.0 ± 6.9	141.7 ± 12.1
7 hours after feeding	174.6 ± 20.4	142.0 ± 6.9	141.7 ± 12.1
After 24 hours	ND	ND	ND

It can be clearly seen that both – the peptide in JP 04282400 and BioZate1 in our study – have the ability to decrease the blood pressure in the SHR model. However, the amount of peptide described in JP 04282400 is approximately 9 times greater than the largest amount having similar efficacy in our study. Even at a much smaller dosage, BioZate 1 is very effective in reduction of blood pressure. The data from JP 04282400 and our results are illustrated in the graphs below. (PBS used as the control in our study is phosphate buffered saline.)

Data reported in Patent No JP 04282400



Data Reported from Our Study



(3) Products of JP 04282400 Prepared by a Procedure Modified to Provide a Fair Comparison Showed Lower Effectiveness for ACE Inhibition Than the Products of the Invention.

An experiment was performed following the methodology described in JP 04282400. We have reproduced the method described in that patent to the best of our ability and have noted variations from it below.

Briefly, our evaluations indicate that profiles of a hydrolysate prepared according to the JP 04282400 teachings and one according to the invention, indicates that the enzyme hydrolysis procedures produce similar profiles of the peptides (see **Figure 4**), but the products of the invention provide distinctly different results in their biological activity against angiotensin converting enzyme.

In brief, the cited Japanese patent describes purification of whey proteins by dialysis, hydrolyzing such purified protein, and isolating the anti-ACE peptides using ion-exchange chromatography. Hydrolysis was performed using trypsin. The hydrolysate was separated from the enzyme by ultrafiltration (UF). JP 04282400 describes isolation of ACE-inhibitory peptide from permeate by using a reverse resin column (ion exchange column). The resulting purified peptide was freeze-dried into powder and was tested for its *in vitro* ACE-inhibitory capacity, *in vivo* (SHR model) capability to reduce blood pressure, and analysis by HPLC.

The method of preparation of the hydrolysate as described in JP 04282400 is shown in **Figure 1**. Modifications to this method were needed as shown in **Figure 2**. The number of water changes during dialysis had to be increased to reduce the lactose content of the end product and to improve the yield and recovery of protein material.

Additional substitutions to the type of reagents and equipment were necessary, as show below, either because JP 04282400 was not specific or because the specific material is no longer available.

- Dialysis tubing: 12,000 – 14,000 MWCO Spectra/Por® (Spectrum Laboratories)
- Trypsin used: Trypsin TPCK from bovine pancreas (Type XIII); 12,700 units/mg protein (Sigma)
- UF-membrane: HP99 Dairy UF Element Specification ST (10 KDa MWCO)

Detailed description of the method

Production of hydrolysate by method described in JP 04282400:

The flow chart of the experiment is shown in **Figure 2**. About 200 g of cheese whey powder containing 10.25% protein was rehydrated in 2 liters of deionized water and dialyzed for 48h. The pH of the dialyzed retentate was adjusted to 8.0 with 1 N NaOH. Calcium content was adjusted to a final concentration of 5 mM. The dialysate was hydrolyzed for 12h using bovine pancreatic Trypsin (Sigma Type XIII). After removing the non-hydrolyzed protein and extraneous matter with pH adjustment to 3.0 and centrifugal separation, the liquid was ultrafiltered (using a 10 kDa MWCO membrane). The permeate collected containing the peptides was freeze-dried. A total of 1.85 g of powder was obtained from this procedure, representing a yield of 0.93% (weight basis). The freeze-dried powder contained 48.5% protein which represents 4.7 x the protein content of the starting material. This confirms that increasing the number of water changes during dialysis indeed reduced the lactose content of the end product and improved the yield of protein recovery. The product is referred to herein as Hydrolysate J

Production of UF-BioZate for comparison:

Hydrolyzed whey protein isolate, BioZate 1, was prepared in the lab following a procedure described in the present application. The enzymatic reaction was stopped by UF using a 30 K Dalton polyethersulfone membrane. The permeate containing the peptides was freeze-dried and was referred to as "UF-BioZate" for comparison against Hydrolysate J produced according to the method described in JP 04282400.

The reverse-resin isolation of the ACE-inhibitory peptides, as described in JP 04282400, (**Figure 3**) was not performed because we are claiming the ACE-inhibitory activity of the entire hydrolysate, and analysis by HPLC of the non-isolated hydrolysates would provide more realistic comparative data.

Leco (Dumas Nitrogen analysis) was used for determining the protein content of the lyophilized powders of the Hydrolysate J. These samples were also analyzed for ACE-inhibitory activity. The lyophilized hydrolysates were analyzed by semi-preparative HPLC method as described below.

Semi-preparative HPLC of BioZate 1 and Hydrolysate J prepared according to the procedure described in JP 04282400.

The HPLC method as described in JP 04282400 (**Figure 3**) could not be reproduced because the type of column described is no longer manufactured. A technically equivalent method of peptide analysis was performed using a semi-preparative HPLC with anion exchange column.

Both hydrolysates (UF-BioZate and Hydrolysate J obtained following the method described in JP 04282400) were fractionated using a semi-preparative HPLC system (Gold Prep 60, Beckman, CA). The equipment had a semi-preparative cell model 166, pump model 126, detector UV/VIS model 166 adjusted at 214 nm, manual injector (2 mL) Rheodyne model 7725 (Cotati, CA), and Gold Nouveau software. A "Q Ceramic HyperD 20 column" (20 x 150 mm; BioSepra Inc., Marlborough, MA) filled with 20 μm beads was used for fractionation of the peptides. The mobile phases used for the elution of components were:

Buffer A: Phosphate buffer (20 mM, pH 8.0)

Buffer B: Buffer A with 1M NaCl

Both buffers were filtered with a 0.2 μm nylon membrane prior to use. Samples were dissolved in Buffer A (15% w/w), filtered with 0.22 μm PVDF membrane, and were injected onto the column. The elution of components was obtained by varying flow rate (black line on chromatogram, Figure 4) and NaCl concentration (shown on chromatograms, **Figures 4A and B**).

For flow rate, the following conditions were used: 1) 0-5 min – 4 mL/min; 2) 5-15 min – 4-7 mL/min; 3) 15-16 min—7 mL/min; 4) 16-28 min—7-28 mL/min; 5) 28-63 min—28 mL/min; 6) 63-68 min—28-4 mL/min. Salt gradient was obtained with the following conditions: 1) 0%, 0-15 min; 2)

0-15%, 15-16 min; 3) 15%, 16-28 min; 4) 15-30%, 28-38 min; 5) 30-100%, 38-40 min; 6) 100%, 40-51 min; 7) 100-0%, 51-53 min.

Results and Discussion:

The method described in JP 04282400 was duplicated to the best of our efforts. The IC_{50} values of the UF filtrate of the hydrolysate described in the patent, Hydrolysate J, and UF-BioZate were determined. Commercially available BioZate 1, without ultrafiltration, was also tested. The comparative results are shown in attached Table 1.

As can be seen from attached **Table 1**, the ACE-inhibitory peptides were isolated in both the UF-BioZate and Hydrolysate J prepared using the method described in JP 04282400. The IC_{50} value of the UF-BioZate is approximately 2.5 times lower than that of Hydrolysate J. This indicates that UF-BioZate has higher ACE-inhibitory activity than Hydrolysate J. Also, commercially produced BioZate 1 samples have lower IC_{50} values than that of Hydrolysate J.

The chromatographic profiles of the two hydrolysates indicates that the enzyme hydrolysis produces similar profiles of the peptides, but distinct differences in their biological activity against angiotensin converting enzyme. One possible explanation for the higher ACE-inhibitory activity of the BioZate 1 and its UF derivative might be the source of the protein composition of the substrate, being obtained from ion exchange processing, used in production of the hydrolyzed products of the invention.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on my information and belief are believed to be true; further that these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of my application or any patent issued thereon.

_____ _____

Anand Rao, Ph.D. Date

Table 1: Values of IC_{50} for synthetic peptide β-lg 142-148, Hydrolysate J obtained from the procedure described in JP 04282400, UF-BioZate, and different lots of commercial BioZate 1. All values shown with standard deviations were from at least triplicate analysis. No standard deviations shown for duplicate analysis (due to limited sample amount).

Product	**Sample Identification**	**IC_{50} Mean[1] ± SD (mg powder/ml)**	**Protein (mg/mg powder)**	**IC_{50} Mean[1] ± SD (mg protein/ml)**
β-lg f142-148	Synthetic peptide	0.25 ± 0.01	1.0	0.25 ± 0.01
Hydrolysate J – produced according to JP 04282400	831-96	2.14[2]	0.485	1.04
UF-BioZate	8301-UF1	0.83 ± 0.03	0.855	0.71 ± 0.026
BioZate 1 - hydrolyzed WPI	LE 001-8-919	1.40 ± 0.04	0.928	1.30 ± 0.037
BioZate 1 - hydrolyzed WPI	LE 001-9-919	1.45 ± 0.03	0.927	1.34 ± 0.027
BioZate 1 - hydrolyzed WPI	LE 001-0-919	1.32 ± 0.05	0.927	1.22 ± 0.046
BioZate 1 - hydrolyzed WPI	LE 002-0-919	1.37 ± 0.02	0.918	1.26 ± 0.018
BioZate 1 - hydrolyzed WPI	LE 003-0-919	1.86 ± 0.05	0.915	1.70 ± 0.046
BioZate 1 - hydrolyzed WPI	LE 004-0-919	1.87 ± 0.06	0.906	1.69 ± 0.054

[1] Mean of at least triplicate analysis.
[2] duplicate analysis

FIGURE 1: Hydrolysis and isolation of hydrolyzed material according to the procedure described in JP 04282400.

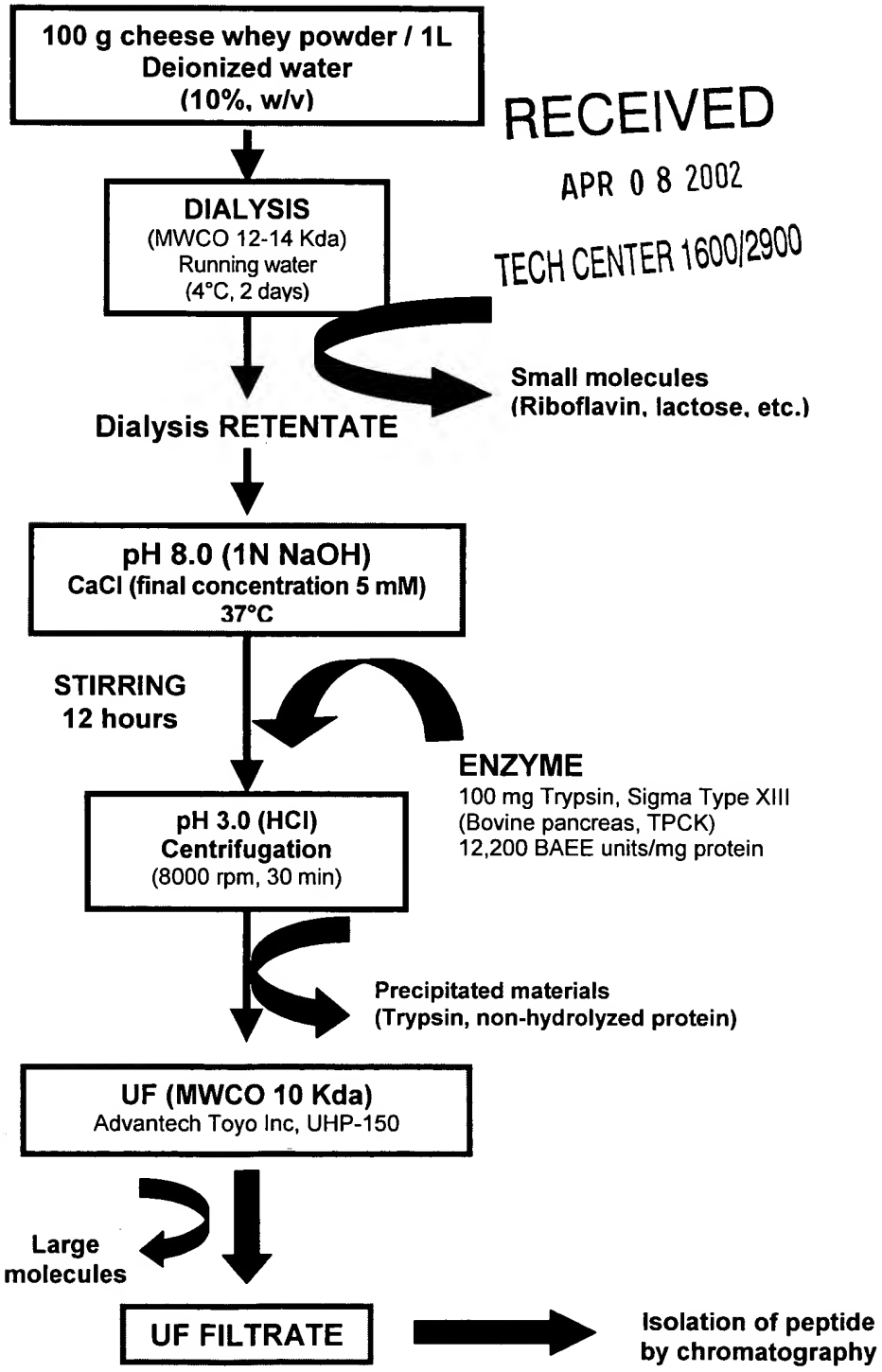


FIGURE 2: Hydrolysis and isolation of hydrolyzed material according to the modified procedure to reproduce the method described in JP 04282400.

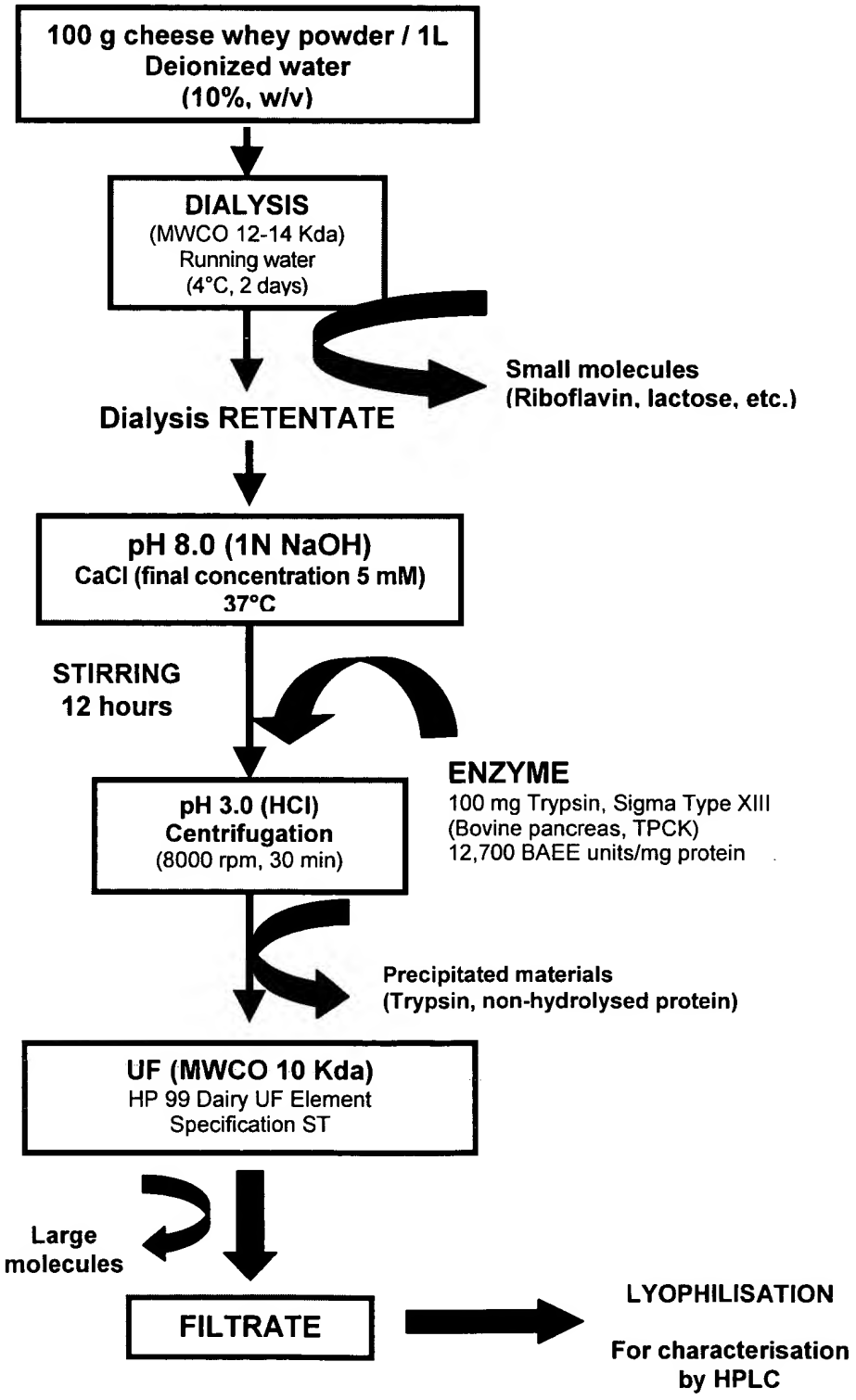


Figure 3: Isolation and analysis of peptide according to the method described in JP 04282400.

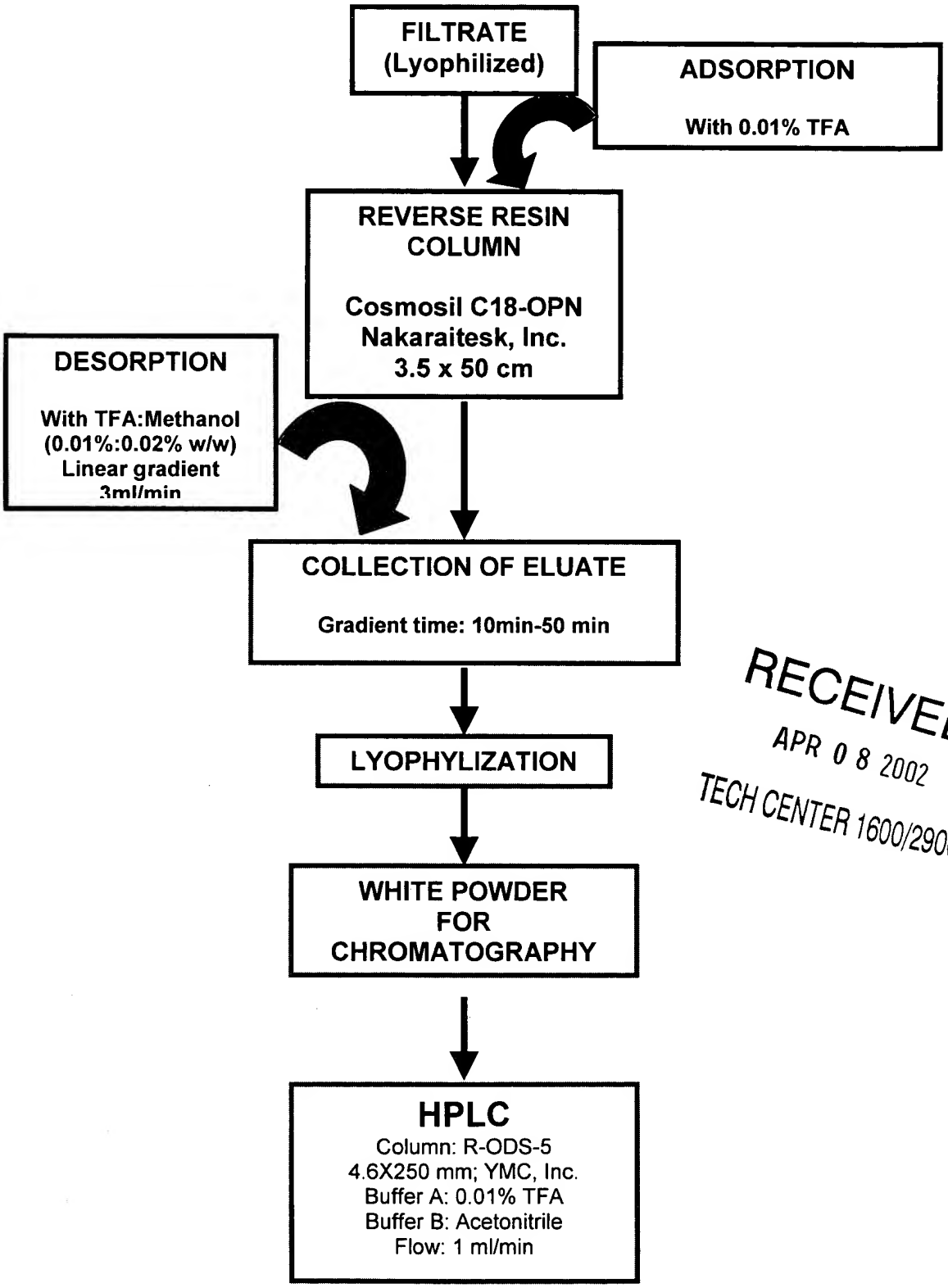


Figure 4: Anion-exchange chromatographic profiles of UF-BioZate (A) and tryptic hydrolysate obtained from JP 04282400 method (B).